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A study of residual lesions in horses that recovered from clinical signs of chronic equine dysautonomia

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Abstract

Background: Equine dysautonomia (ED) causes degeneration and loss of autonomic neurons. Approximately 50% of chronic cases recover, but it is unclear how they survive neuronal loss.

Objectives: To assess lesions, autonomic neuron numbers, interstitial cells of Cajal (ICC), and neurodegeneration in recovered cases.

Animals: Thirteen cases (group ED), euthanized 10.3 ± 5.2 (1–16) years from diagnosis and 6 age-matched controls (group C).

Methods: Prospective, case control; routine post mortem examination, neuron counts in peripheral and enteric ganglia and immunohistochemical assessment of neural networks (Protein gene product [PGP] 9.5), ICC (c-kit), and neurodegeneration (beta-amyloid precursor protein and ubiquitin) in intestine.

Results: Postmortem findings in group ED were small intestinal dilation (4/12, 33%) and muscular hypertrophy (4/12, 33%), and gastric mucosal hypertrophy (3/11, 27%) and ulceration (4/11, 36%). Neuron density was lower in group ED (mean 39% lower for cranial cervical ganglion [$P < .001$], median 44% lower in celiacomesenteric ganglion [$P = .01$]). In intestine, neuronal depletion was worst in ileum (median 100% lower in submucosal plexus [$P < .001$], 91% lower in myenteric plexus [$P = .004$]). Group ED had less PGP 9.5 staining in ileal myenteric plexus (mean 66% lower [$P = .04$]) and circular muscle (median 75% lower [$P = .006$]). In ileum, there was less c-kit staining in myenteric plexus (median 57% lower [$P = .02$]) but not *muscularis externa*. Beta-amyloid precursor protein and ubiquitin results were not indicitive of neurodegeneration.

Conclusions and Clinical Importance: Intact ICC in *muscularis externa* might help maintain motility after neuronal loss. Treatment supporting ICC function warrants investigation.

KEYWORDS

BAPP, c-kit, dysautonomia, grass sickness, horse, interstitial cells of Cajal, intestinal motility, PGP 9.5, ubiquitin

Abbreviations: BAPP, beta-amyloid precursor protein; CCG, cranial cervical ganglion; CM, circular muscle; CMG, celiacomesenteric ganglion; ED, equine dysautonomia; HE, hematoxylin and eosin; IHC, immunohistochemistry; LM, longitudinal muscle; LVC, left ventral colon; MP, myenteric plexus; PGP 9.5, protein gene product 9.5; SC, small colon; SMP, submucosal plexus.

1 | INTRODUCTION

Equine dysautonomia (ED; equine grass sickness) is a commonly fatal disease of horses characterized by autonomic nervous system dysfunction. It occurs predominantly in northern Europe[1] and South America.[2] The cause is unknown, although current theories include toxicoinfection with *Clostridium botulinum* type C[3] or a pasture-derived mycotoxicosis.[1,4] Pathological changes include neuronal degeneration in the autonomic ganglia, enteric nervous system, brainstem, and spinal cord nuclei.[5-7] Although acute and subacute ED are fatal, over 50% of chronic cases can survive with supportive management.[8] Clinical signs of acute and subacute ED include gastrointestinal stasis, dysphagia, and nasogastric reflux. Impaired gastrointestinal motility can also be present in chronic ED but does not cause complete stasis and such cases present with cachexia, mild colic, dysphagia, and hyporexia. Many survivors return to work after many months; however, some retain long-term residual deficits, including mild dysphagia and recurrent colic.[9,10]

In chronic ED, the number of neurons in prevertebral and paravertebral ganglia[11] and enteric plexuses[6,12] is lower than in acute ED, and loss is most severe in the ileum.[6,10] However, the means by which chronic cases survive despite neuronal depletion is not established.

More recently, attention has turned to the interstitial cells of Cajal (ICC), a cellular network present mainly in the intestinal *muscularis externa* and myenteric plexus (MP). Interstitial cells of Cajal are considered to have a pacemaker function in initiating slow wave activity.[13] Although ICC numbers are reduced in ED, this reduction was significantly less in circular muscle (CM) of chronic compared with acute cases[14] and in vitro electrical activity can still be prominent in chronic ED, suggesting that ICC-associated pacemaker activity remains intact despite neuronal loss, although that study did not examine recovered cases.[15] This was supported by the study of a long-term recovered case with severe neuronal loss in ganglia and ileal plexuses but an apparently intact network of ICC in the MP and CM.[16]

The number of chromatolytic neurons in recovered cases is small on standard histological examination.[10] However, it remains unclear whether more subtle evidence of neuronal dysfunction might be revealed through the assessment of various marker proteins, known to change in human neurodegenerative diseases and acute and subacute ED. There is an increase in beta-amyloid precursor protein (BAPP), a protein associated with misfolding/aggregation, and changes in intracellular localization of ubiquitin (ubiquitin carboxy-terminal hydrolase L1) which regulates protein misfolding, in the neurons of acute and subacute ED cases.[17-19] Expression of these proteins in long-term survivors has not yet been studied.

The objectives of the present study were to determine the gross lesions, quantify the degree of neuronal depletion in the ganglia and enteric plexuses, determine the extent of the ICC network, and quantify markers of neurodegeneration in long-term recovered cases of ED in order to better understand how horses can recover. It was hypothesized that the ICC might help maintain intestinal function when marked neuronal depletion is present in recovered cases, but that evidence of ongoing neurodegeneration would be minimal.

2 | MATERIALS AND METHODS

2.1 | Animals

In this prospective study, inclusion criteria for ED cases consisted of a diagnosis made on the basis of detailed clinical examinations by experienced clinicians at the Equine Hospital, at least 1 year between diagnosis and euthanasia, availability of at least 2 tissues (including the ileum and at least 1 prevertebral or paravertebral ganglion), and clear post mortem histological evidence of neuronal loss in the ileum typical of chronic ED on standard hematoxylin and eosin (HE) staining. *Antemortem* diagnosis by invasive intestinal biopsy was not undertaken as this is considered to adversely affect outcome in some cases,[9] and in view of the typical clinical signs, it was not considered necessary. Recovery was defined as an increase to normal (or near normal) weight, lack of, or marked improvement in clinical signs so that nursing was no longer required, and ability to undertake exercise (mainly ridden work). Cases were excluded if they did not meet these criteria or if autolysis was advanced. Controls comprised 6 age-matched horses euthanized at the Equine Hospital for reasons unconnected with ED, gastrointestinal or neurological disease. The study was approved by the local Veterinary Ethical Review Committee (approval no. 118/15), and owner consent was obtained for inclusion of their animals in the study. All horses were euthanized on the basis of clinical need and not solely for the purpose of the study. Euthanasia was carried out by barbiturate overdose.

2.2 | Postmortem examination and tissue collection

All cases and controls were subjected to full gross postmortem examination at the Veterinary Pathology Unit on the day of euthanasia and usually immediately after death. In 1 additional case (ED10), tissues were supplied by the primary care veterinarian and had been collected the day after euthanasia. A gross description was recorded. Intestinal dilation was defined as increased diameter with a turgid wall and was considered distinct from dilation occurring due to antemortem or postmortem gas accumulation. Esophageal and intestinal muscular hypertrophy was defined as thickening of the *muscularis externa* compared with adjacent areas of the organ and in comparison with the expected normal appearance. Whenever possible, tissues collected were cranial cervical ganglion (CCG), thoracic sympathetic chain intervertebral ganglia, celiacomesenteric ganglion (CMG), jejunum, ileum, left ventral large colon (LVC), and small colon (SC); however, for various reasons, not all tissues were available for every case. In some cases, esophagus, stomach (*margo plicatus*), duodenum, cecum, pelvic flexure of the large colon, rectum, adrenal gland, pituitary gland, brain, spinal cord, triceps muscle, bladder, liver, heart, kidney, and spleen were also collected.

2.3 | Routine histopathology

Tissues were placed in 10% phosphate-buffered formalin (pH 7.4) until fixed, then processed to paraffin wax blocks, from which 4 μm thick sections were cut and stained with HE using standard

techniques. Whenever possible, intestinal tissues were oriented, so sections were cut parallel to the long axis of the CM smooth muscle cells. The HE sections were used to assess general pathological and artefactual changes, to assess neuron morphology, and to count neurons in the CCG, CMG, jejunum, ileum, LVC, and SC. The slides were scanned in a Nanozoomer slide scanner (Hamamatsu Ltd, Welwyn Garden City, United Kingdom). Using the scanned images of CCG and CMG, 4 random 1 mm^2 were created and the average number of neurons per mm^2 counted using ImageJ software (National Institutes of Health). Random images were used to allow for the fact that neurons are not uniformly distributed in ganglia. In the sections from the 4 parts of the intestine, the length of the section was instead measured on the scanned slide and the number of neurons per centimeter counted separately for the submucosal plexus (SMP) and MP from the original glass slides on which small neurons were more easily identified using an Olympus BX51 microscope (Olympus Ltd, Southend-on-Sea, United Kingdom).

2.4 | Immunohistochemistry

Immunohistochemistry was carried out on fixed paraffin wax-embedded sections (4 μm) on SuperFrost Plus coated slides (Thermo Electron; Runcorn, Cheshire, United Kingdom) that were dewaxed and rehydrated. For protein gene product 9.5 (PGP 9.5), the primary antibody was rabbit anti-human polyclonal antibody Clone UCHL1 (Cat no 7863-0504; Bio-Rad, Kidlington, United Kingdom) diluted 1/2000. After incubation at 4°C overnight, secondary antibody (goat anti-rabbit HRP 1/50, Cat no PI-1000; Vector Laboratories, Peterborough, United Kingdom) was applied and incubated for 30 minutes followed by visualization with a commercial immunolabeling kit (DakoCytomation EnVision+ System-HRP, DAB K4001; Dako, Ely, United Kingdom). Beta-amyloid precursor protein staining was carried out after high pH antigen retrieval overnight at 60°C. Primary antibody was monoclonal mouse anti-APP A4 clone 22C11 (Cat no MAB348, Merck), diluted 1/16000 and incubated for 120 minutes at room temperature followed by incubation with anti-mouse antibody-HRP (EnVision+, Dako) for 40 minutes and visualization with DAB. For c-kit, antigen retrieval was carried out using 0.01 M citrate pH 6.0 at 110°C for 5 minutes. The primary antibody (rabbit polyclonal anti-CD117, Cat no A4502, Dako) was diluted 1/200 and incubated overnight at 4°C followed by anti-rabbit antibody-HRP (EnVision+; Dako) for 40 minutes and visualization with DAB. For ubiquitin, antigen retrieval was carried out using citrate buffer pH 6 at 110°C for 5 minutes. The primary antibody (monoclonal mouse antibody, clone Ubi-1, Cat no MAB1510; Millipore, Watford, Hertfordshire, United Kingdom) was diluted 1/35000, and sections were incubated for 30 minutes at room temperature. This was followed by incubation for 40 minutes at room temperature with secondary antibody (anti-mouse antibody-HRP, EnVision+; Dako) and visualization with DAB. All sections were counterstained with hematoxylin. The immunohistochemistry (IHC) methods have been previously validated for equine tissues in our laboratory.[16,18,19]

The method of analyzing the results of IHC in intestine was chosen based on the stain, site, and morphology of the stained areas. The slides of jejunum, ileum, LVC, and SC stained with PGP 9.5 and c-kit were scanned in the Nanozoomer scanner. Two random x100 images of well-oriented, straight regions (covering most of the well-oriented areas) were taken of each of the MP, CM, and for ileum only, the longitudinal muscle (LM), and the percentage area stained was determined for each image to calculate the average percentage area stained per site, using ImageJ software. This was accomplished by splitting the image, selecting the blue channel, and converting it into binary for particle analysis. The threshold was set by comparison of multiple typical images with the original IHC and was kept at the same level for all images for each stain. Longitudinal muscle was only assessed in the ileum due to the small amount of staining and thinness of the muscle layer in the other parts of the intestine. For the MP, the 2 x100 images were created by marking and cropping a rectangle along the length and width of the MP, in random areas with straight orientation of the MP, similar to a method previously described.[20] The SMP was not studied for PGP 9.5 or c-kit due to minimal staining and artefactual disruption of the loose submucosal connective tissue preventing reliable assessment per unit area.

Beta-amyloid precursor protein staining of neurons and axons was assessed semi-quantitatively as the percentage stained and intensity of staining in the CCG, thoracic chain, and CMG, and in the SMP and MP of the jejunum, ileum, LVC, and SC as follows: %positive: 0 = completely negative, 1 = 1%-25% of cells positive, 2 = 26%-50% positive, 3 = 51%-75% positive, and 4 = >75% positive; intensity score of neurons: negative (0), weakly positive (1), moderately positive (2), and strongly positive (3); intensity score of axons; negative (0), moderately positive (1), and strongly positive (2).

Ubiquitin staining was assessed separately in the cytoplasm and nucleus of neurons in the CCG, thoracic chain, and CMG, and in the SMP and MP of the jejunum, ileum, LVC, and SC, including the SMP and MP of the 4 intestinal tissues. The intensity score in the cytoplasm and nucleus of neurons and in axons was classified as negative (0), weakly positive (1), moderately positive (2), and strongly positive (3).

Semi-quantitative staining for BAPP and ubiquitin was carried out in preference to image analysis as intensity of staining is not linear using DAB-based visualization. Beta-amyloid precursor protein and ubiquitin slides were examined blind and scored twice on different days by the same pathologist (E.M.) for consistency. Unlike for c-kit and PGP 9.5, assessment of BAPP and ubiquitin was possible in the SMP despite artefactual disruption, because the assessment was based on morphology of individual neurons rather than percentage area positively stained.

2.5 | Statistics

All results were assessed for normality using the Ryan-Joiner test. The neuron counts and IHC score results were compared between ED and control groups for each measurement using unpaired *t* tests for parametric data and the Mann-Whitney *U* test for nonparametric data. Statistical analysis was carried out using Minitab v17 (Minitab Ltd, Coventry, United Kingdom), and statistical significance was set at $P \leq .05$. The main data analyses that are not presented in the manuscript are available in the Supporting Information.

3 | RESULTS

3.1 | Animals

An adequate range of tissues was available from 13 horses considered to have recovered from ED. These were obtained postmortem between 2002 and 2017. In 1 (ED10), only neuron counts could be carried out as autolysis decreased the quality of IHC.

The horses in the ED group consisted of 10 neutered males and 3 females of various breeds; the age at euthanasia was 15.3 ± 4.8 (mean ± SD) years (range 7-20 years), and the time between onset and euthanasia was 10.3 ± 5.2 years (1-16 years). Group C comprised 2 neutered males and 4 females of several breeds, and the mean age at euthanasia was 19.3 ± 6.6 years (10-28 years). There was no significant difference between the ages of groups ED and C (P = .20). Details of signalment are shown in Supplementary Table 1. All ED cases had shown typical clinical signs of ED at the onset. In the period shortly before euthanasia, the main clinical sign in the 9 cases for which information was available was recurrent colic (3/9), and the owners requested euthanasia for that reason. However, it should be noted that recurrent colic had not been a major feature until then. All other cases were euthanized for a variety of reasons unrelated to ED, and some had performed at a high level of athleticism including racing and eventing (Supplementary Table 2).

3.2 | Gross findings and histopathology

The main findings relating to the digestive tract on gross postmortem and histological examination are presented in Supplementary Table 2. These were dilation (4/12, 33%) and muscular hypertrophy (4/12, 33%) of the small intestine, especially the distal jejunum and the ileum, and gastric mucosal hypertrophy (3/11, 27%) and ulceration (4/11, 36%). Of 6/12 (50%) horses with either dilation or muscular hypertrophy of the small intestine, 3 had a history of recurrent colic, 2 had no history of colic, and in 1, the clinical signs were unknown. Liver changes included mild periportal infiltration, mainly with small lymphocytes (4/7 ED cases), but this was also present in 3 of 6 controls. No abnormalities were evident in other sites other than those related to the reason for euthanasia (eg, laminitis, arthritis). In group C, there were no gross or microscopic gastrointestinal tract abnormalities of importance. The neurons in the CCG, thoracic chain, and CMG of group ED appeared subjectively depleted on routine assessment, but chromatolytic neurons were rare; there was a corresponding increase in support cells and trabecular structures (Figure 1A). No abnormalities were detected in the ganglia in group C (Figure 1B) apart from occasional chromatolytic neurons.

3.3 | Neuron counts

The neuron counts in the CCG, CMG, and the SMP and MP of the jejunum, ileum, LVC, and SC are presented in Figures 2 and 3, and Supplementary Table 3. The number of neurons in the CCG and CMG was significantly lower in the ED group (mean 39% lower in the CCG [P < .001] and median 44% lower in the CMG [P = .011]). This supported the original diagnosis and confirmed that full regeneration had not occurred. The ileum was the worst affected part of the intestine with markedly reduced neuronal numbers in the SMP and MP (median 100% lower in the SMP [P < .001] and 91% lower in the MP [P = .004]). In the SMP and MP, 11 of 13 (85%) and 3 of 13 (23%) cases had ≤1 neuron per centimeter, respectively. The jejunum was affected to a lesser degree with significantly lower numbers in the SMP only (median 59% lower [P = .02]). Neuron numbers in the large intestine were not significantly different from controls.

3.4 | Immunohistochemistry

When sections were stained for PGP 9.5 for nerve networks, the percentage area stained in the MP (mean 66% lower [P = .04]) and CM (median 75% lower [P = .006]) of the ileum was significantly less in ED cases compared to controls (Figure 1C, D and Supplementary Table 4). No significant difference was evident in the LM of the ileum or the MP or CM of the jejunum, LVC or SC in ED cases compared with controls.

The results of staining for c-kit to demonstrate the ICC are presented in Figures 4 and 5 and Supplementary Table 5. The ICC in the ileum were significantly lower in group ED than the controls in the MP (median 57% lower [P = .02]) but not the CM or LM. The ICC were also significantly depleted in the MP (median 42% lower [P = .04]) and CM (mean 66% lower [P = .003]) of the SC compared to control cases. There was evidence for apparently intact networks of ICC in the *muscularis externa*, with cytoplasmic extensions running parallel to the smooth muscle cells (Figure 1E, F).

In the jejunum and ileum, nuclear staining intensity for ubiquitin was lower in the SMP and MP in ED compared to control cases (jejunal SMP [P = .03], jejunal MP [P = .03], ileal SMP [P = .04], and ileal MP [P = .03]; Supplementary Table 6).There were no significant differences in nuclear or cytoplasmic staining for ubiquitin in the CCG, thoracic chain, CMG, or the SMP or MP of the LVC or SC in group ED compared with group C (Figure 6A, B).

Beta-amyloid precursor protein staining (percentage of neurons stained and intensity of staining) was not significantly higher in the ganglia (Figure 6C, D) or the MP or SMP of the jejunum, ileum, LVC, or SC of ED cases compared with controls. In contrast, the percentage of neurons stained and intensity of BAPP staining in the SMP of the ileum were significantly lower in ED cases (percentage stained, P = .03, intensity of staining, P = .03). There was no significant difference between the groups for BAPP staining of axons (Supplementary Table 6).

As some tissue blocks from the ED cases were up to 16 years old, it was initially considered that prolonged storage might have affected the IHC staining, but no correlation was found between the age of block and intensity of staining (data not shown).

4 | DISCUSSION

The major findings in this study were that in long-term recovered ED cases, gross postmortem findings included small intestinal dilation and

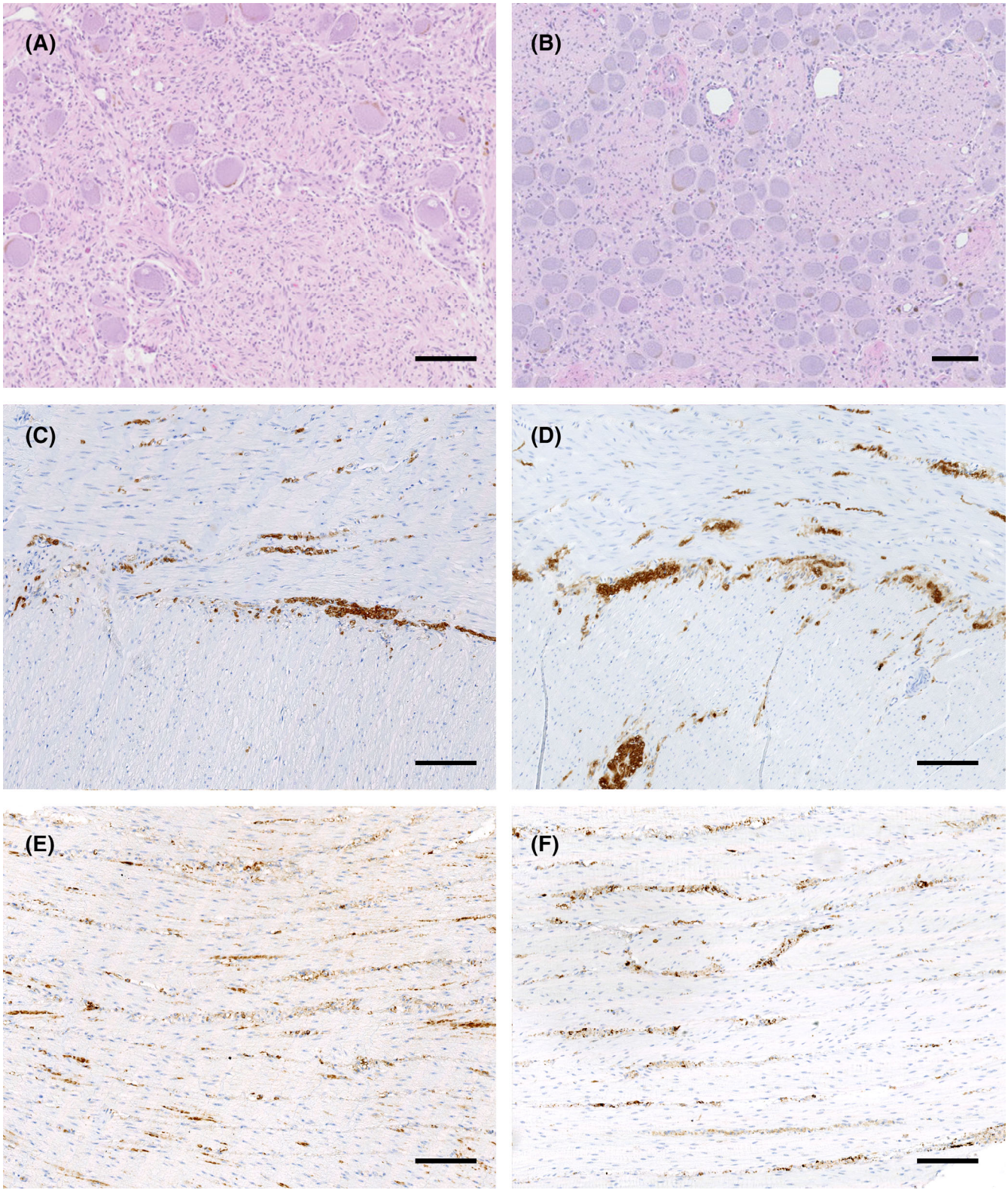

FIGURE 1 Celiacomesenteric ganglion (CMG) from case ED6 with a reduced density of neurons, hematoxylin and eosin (HE; scale bar 100 μm) (A); CMG from control case C2 with a normal number of neurons, HE (scale bar 100 μm) (B); Ileum from case ED7 with a reduction in nerve networks in the circular muscle (CM), myenteric plexus (MP), and longitudinal muscle (LM; from the top to bottom of image); protein gene product 9.5 [PGP 9.5] (scale bar 50 μm) (C); Ileum from control case C3 with normal nerve networks in the CM, MP, and LM; PGP 9.5 (scale bar 50 μm) (D); Ileum from case ED7 with a normal network of interstitial cells of Cajal (ICC) in the CM, c-kit (scale bar 50 μm) (E); Ileum from control case C2 with a normal network of ICC in the CM, c-kit (scale bar 50 μm) (F)

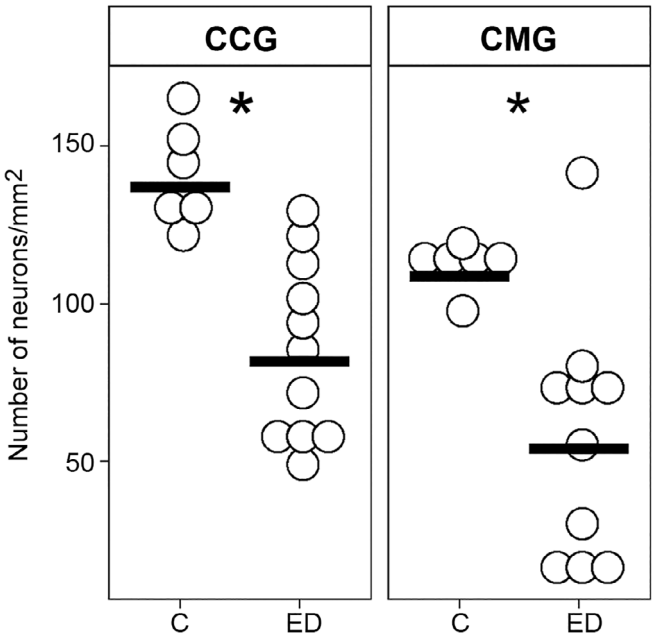

FIGURE 2 Individual value plot of neuron numbers in the cranial cervical and celiacomesenteric ganglia of groups ED and C. C, control group; ED, equine dysautonomia group; CCG, cranial cervical ganglion; CMG, celiacomesenteric ganglion; bar, mean value. *P < .001 for CCG (unpaired t test) and P = .011 for CMG (Mann-Whitney U test)

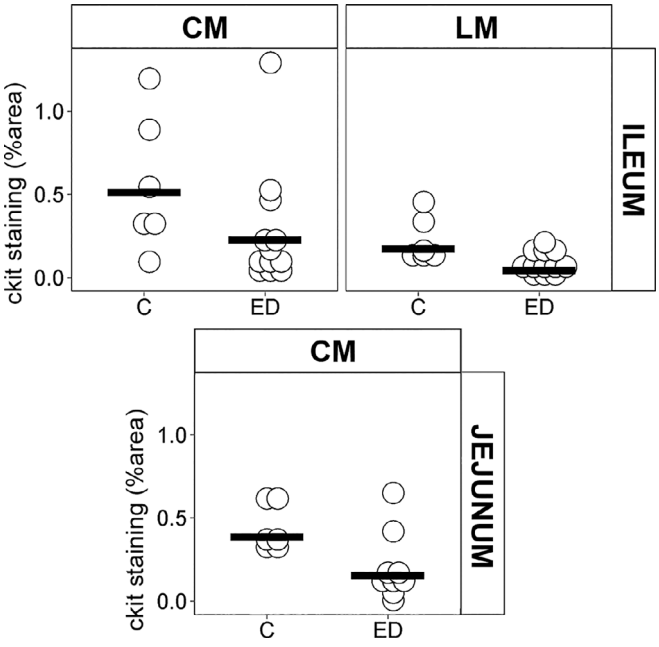

FIGURE 4 Individual value plot of c-kit staining in the CM of the jejunum and ileum, and the LM of the ileum of groups ED and C. C, control group; ED, equine dysautonomia group; CM, circular muscle; LM, longitudinal muscle; bar, mean value. P > .05 for all comparisons between groups

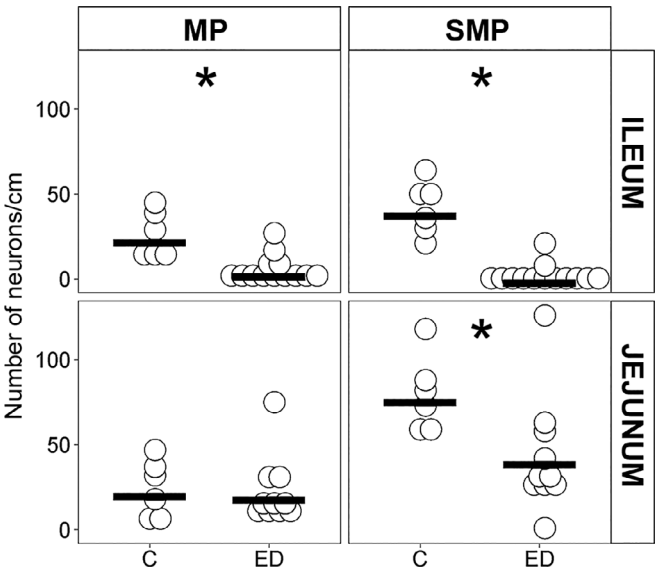

FIGURE 3 Individual value plot of neuron numbers in the submucosal and myenteric plexus of the jejunum and ileum of groups ED and C. C, control group; ED, equine dysautonomia group; SMP, submucosal plexus; MP, myenteric plexus; bar, mean value. *P = .004 for ileum MP, P < .001 for ileum SMP, P = .023 for jejunum SMP (Mann-Whitney U test)

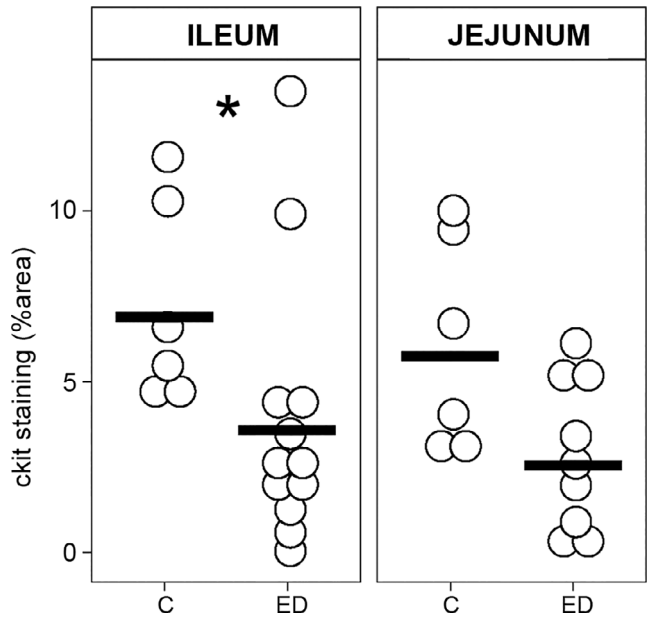

FIGURE 5 Individual value plot of c-kit staining in the myenteric plexus of the jejunum and ileum of groups ED and C. C, control group; ED, equine dysautonomia group; bar, mean value. *P = .02 for ileum MP (Mann-Whitney U test)

muscular hypertrophy, and gastric mucosal hypertrophy (as assessed by gross and histological examination) and ulceration. Density of neurons in prevertebral and paravertebral ganglia was significantly lower in group ED and in the intestinal tract, and the ileum was worst affected, especially the SMP. In the ileum, group ED had significantly less PGP 9.5 staining for neural networks in the MP and CM than controls, and there was also less staining for c-kit (ICC) in the ileal MP but not the *muscularis externa*. No such changes occurred in the large

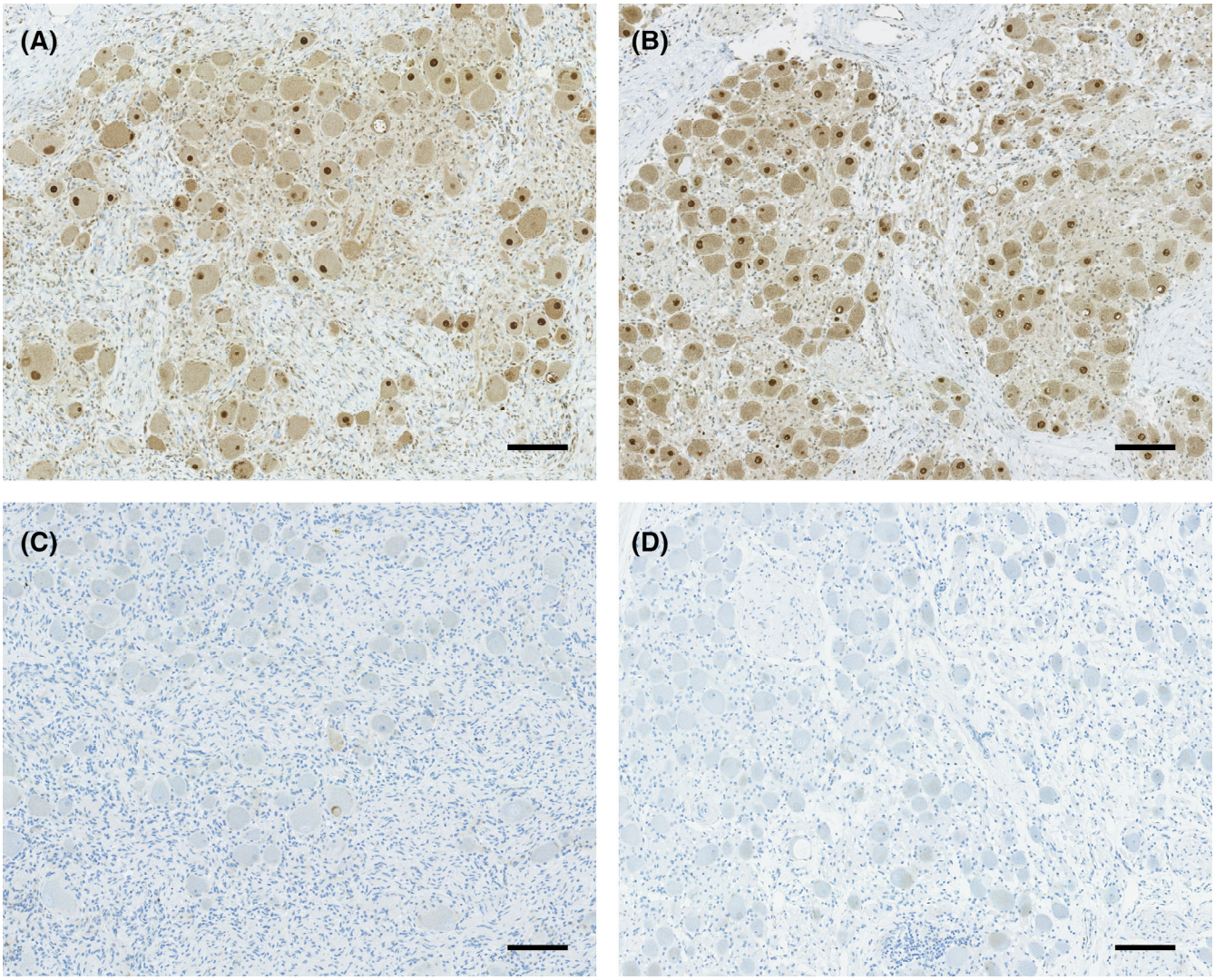

FIGURE 6 Celiacomesenteric ganglion (CMG) from case ED13 with normal nuclear and cytoplasmic staining of neurons for ubiquitin (A); CMG from control case C4 with normal staining for ubiquitin (B); CMG from case ED13 with normal low staining of neurons for beta-amyloid precursor protein (BAPP) (C); CMG from control case C4 with normal low staining for BAPP (D). Scale bars 50 μm

intestine. Beta-amyloid precursor protein and ubiquitin did not show consistent changes suggestive of neurodegeneration. The results suggest that despite considerable loss of neurons in peripheral ganglia and small intestine, the ICC network in the *muscularis externa* of the small intestine is adequate. However, there is no evidence for ongoing neurodegenerative changes as assessed by BAPP and ubiquitin expression. The ICC could provide a means for maintaining intestinal motility in horses that have recovered from ED.

The most common residual clinical signs in recovered cases of ED were recurrent mild colic and terminal severe colic, as previously described.[9,10] This is consistent with dilation sometimes combined with hypertrophy of the *muscularis externa*, especially of the distal jejunum and the ileum. However, 2 horses with ED had dilated small intestine but no history of colic; therefore, either mild clinical signs can be missed or the dilation per se is not painful in some cases. Dilation can be an incidental finding or associated with postmortem gas accumulation, but the macroscopic appearance of dilated areas in ED cases was considered distinct from incidental changes. The degree of dilation with turgidity of the wall and the muscular hypertrophy observed suggested a probable chronic course, and it is possible that, in some horses, age-related loss of neurons might have contributed to preexisting disease-related neuronal loss, resulting in a critical threshold of neuronal depletion, beyond which adequate intestinal motility could not be maintained. However, this remains speculative. Hypertrophy of the *muscularis externa* could represent a compensatory response to motility disturbances resulting from neuronal loss.[21] It is also feasible that muscular hypertrophy contributed to intestinal dilation in cases with dilation immediately proximal to an area of hypertrophy/stricture or dysmotility. Ulceration of the squamous gastric mucosa and hypertrophy of the glandular mucosa was common and again might have resulted from poor motility resulting in delayed gastric emptying. Although muscular hypertrophy of the ileum and gastric ulceration can have other causes, association with ED was considered likely in view of their frequency. However, association with ED is not

proven in the present study, especially as the group sizes were small. As expected, and consistent with the fact that large colon impaction is a feature of acute and subacute rather than chronic ED, abnormalities related to ED were not detected on gross or routine histological examination of the large intestine. Similarly, IHC revealed minimal changes in the large intestine in the recovered cases. A mild predominantly periportal inflammatory infiltrate in the liver can occur in ED[22]; however, in our study, this was present to a similar extent in control horses and is most likely to be age-related.

As previously described in ED cases at the time of clinical disease[6,11,12] and in the few recovered cases previously studied,[10] neurons were also consistently depleted in horses after clinical recovery from ED. Thus, our study revealed neuronal depletion in recovered ED cases in the CCG and CMG, the SMP and MP of the ileum, and, to a lesser extent, the SMP and MP in the jejunum. The degree of neuronal loss in the ileum was marked and included horses that had few or no residual clinical signs. The results of PGP 9.5 staining for nerve networks complemented the neuron counts in that a significant reduction in expression was present in the MP of the ileum. It was also clear that neuron counts in the LVC and SC were either unaffected or affected to a much lesser degree.

Protein gene product 9.5, the enzyme ubiquitin carboxy-terminal hydrolase L1, is primarily used to demonstrate neural networks. Protein gene product 9.5 is also a cofactor for ubiquitin and together they form part of the ubiquitin proteasome system. This pathway is involved in degradation of nonessential and damaged proteins including BAPP[23] and is defective in a variety of human neurodegenerative diseases including Alzheimer's and Parkinson's diseases, spinal muscular atrophy, and amyotrophic lateral sclerosis.[24-27] There is a decrease in PGP 9.5 expression in both prevertebral and paravertebral ganglia from acute and subacute ED, whereby it was hypothesized that it could contribute to neuronal degeneration.[17] Our findings are not inconsistent with this hypothesis, but it is perhaps more likely that reduced PGP 9.5 expression might simply reflect loss of neurons and nerve networks. Regarding ubiquitin, an increase in cytoplasmic and decrease in nuclear expression occurs in the CCG of acute and subacute ED cases.[18] Consistent with these reports, we also identified reduced nuclear staining intensity for ubiquitin in the SMP and MP in both jejunum and ileum in ED compared to control cases. This could reflect the previously reported abnormalities of the ubiquitin proteasome system in ED,[18] which might ultimately contribute to the gradual decompensation of enteric function in some cases.

Amyloid β peptide is a cleavage product of BAPP that accumulates in the extracellular space in Alzheimer's disease.[28] Beta-amyloid precursor protein increases in the perikarya of CCG neurons in acute and subacute ED and is thought to be involved in the pathogenesis of ED.[18] The increase in BAPP staining is also of value in the antemortem diagnosis of acute and subacute ED, and there is increased expression in rectal biopsies from ED-affected horses.[19] In contrast, we found no evidence of significantly greater BAPP staining at any site in the ED cases compared with controls; therefore, accumulation of BAPP does not appear to be a feature of recovered ED cases. This suggests that neuronal degeneration occurs during the earlier stages of the disease but is not an ongoing process in recovered cases.

Of particular interest in the context of maintenance of intestinal motility were the ICC. C-kit, a receptor tyrosine kinase, is commonly used as a marker of ICC in a range of species. It is also expressed by mast cells, but the typical morphology of the ICC and the usual absence of mast cells from the MP, CM, and LM support the use of c-kit as an ICC-specific marker in the context of this study. The ICC numbers were significantly lower in the ileal MP in the ED group, compared with the controls but were not significantly lower in the ileal CM and LM. Interstitial cells of Cajal were also not significantly depleted in the MP or CM of the jejunum. These findings create the intriguing possibility that the presence of an adequate network of ICC, at least in the *muscularis externa*, could have a role in maintaining small intestinal motility in the face of marked neuronal depletion.[16] Interstitial cells of Cajal were also significantly depleted in the MP and CM of the SC in ED cases, but the number of samples from this site was small, increasing the risk of type 1 errors and raising uncertainty over its importance.

Considerable research is currently focused on the function of the ICC and their role in human gastrointestinal diseases. The ICC are mesenchymal in origin and share a common progenitor with smooth muscle cells. They form a branching network of cells in all layers from the submucosa to the serosa, including the muscle layers and MP area, from the esophagus to the internal anal sphincter. In the CM and LM, the cells of the ICC run parallel to the orientation of the smooth muscle cells[29] and have long fiber-like projections. Interstitial cells of Cajal are known to have a role as “pacemaker cells” and are responsible for the generation of slow waves in the intestine of many species, including humans[30] and horses.[14] In comparison, smooth muscle cells can show spontaneous activity but cannot generate slow wave activity on their own.[31] The ICC form part of a syncytium involving smooth muscle and neurons[13,29] and abolition of ICC prevents slow waves in some human motility disorders.[32-34]

In horses, ICC occur in the MP, CM and, in the ileum, the LM.[20,35] Reduction in ICC in ileum and large colon occurs in ED, but in ileal CM, this reduction is less in chronic than acute cases.[14] in vitro electrical activity can still be prominent in ED,[15] a finding that may support the continued presence of ICC-associated pacemaker activity in the face of neuronal loss.[15] It is therefore possible that the neuronal loss in ED may not completely prevent propagation of electrical events from the ICC to the muscular layers, thus allowing continued peristaltic activity in recovered cases. This is consistent with the proposal that depletion or malfunction of 1 component of the gastrointestinal neuromuscular system (ICC, neural, or muscular) does not necessarily result in failure of the whole system, due to a degree of overlap in function and the capacity for other components to compensate.[34] Despite this potential for a compensatory response, it is likely that a critical threshold exists, beyond which no further compensation is possible, thus leading to chronic intestinal remodeling and sometimes severe colic that may prompt euthanasia on humane grounds. These proposals might explain how recovered ED cases can apparently maintain function for many years but can ultimately succumb to

critical failure, that is, acute colic requiring euthanasia. Furthermore, ICCs possess the capacity to regenerate which could help to prolong motility following depletion of ICC.[36,37] In view of this, the ICC are considered a promising target for the development of drug therapies designed to maintain their function, as has been proposed in human medicine.[38]

This study presented a number of challenges; in particular, obtaining a sufficient number of well-documented recovered ED cases that returned to the Equine Hospital from a wide geographical area for postmortem examination, often many years after the initial diagnosis. The original diagnoses were made by experienced clinicians on the basis of typical clinical signs. Ideally biopsies would have been undertaken to confirm antemortem diagnosis, but this was considered undesirable and unnecessary on clinical grounds, and marked reduction of neurons evident many years later supports the original diagnosis. As the study was conducted over a 15-year period, the study's pathologist was not always available to collect samples from every case; as a result, some samples were missing from some cases. However, this still represents the largest and the most detailed study undertaken to date examining the pathology of ED cases that had recovered for such a long time period.

In conclusion, the main findings during gross postmortem examination of long-term ED survivors were gastric ulceration, small intestinal dilation, and hypertrophy of the *muscularis externa*, which appeared to be of a prolonged duration. In some of the cases, these findings were associated with chronic recurrent colic or a terminal severe colic episode. Recovered cases also had evidence of significant neuronal loss in prevertebral and paravertebral ganglia and in the enteric plexuses of the small intestine, particularly the ileum. However, the ICC networks are mainly intact in the *muscularis externa* of the small intestine which may contribute to the maintenance of intestinal motility in such cases. If therapeutic strategies designed to support ICC function could be developed in the future, this might be of value in the treatment of chronic or recovered cases of ED.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee (approval no. 118/15).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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REFERENCES

1. Pirie RS, Jago RC, Hudson NPH. Equine grass sickness. *Equine Vet J*. 2014;46:545-553.
2. Uzal FA, Robles CA, Olaechea FV. Histopathological changes in the coeliaco-mesenteric ganglia of horses with 'mal seco', a grass sickness-like syndrome, in Argentina. *Vet Rec*. 1992;130:244-246.
3. Hunter LC, Miller JK, Poxton IR. The association of *Clostridium botulinum* type C with equine grass sickness: a toxicoinfection. *Equine Vet J*. 1999;31:492-499.
4. Doxey DL, Robb J, Milne EM, Gilmour JS. Mycological studies on the equine intestinal tract with particular reference to equine dysautonomia (grass sickness). *Ann Appl Biol*. 1990;117:337-341.
5. Obel L. Studies on grass disease. *J Comp Pathol*. 1955;65:334-354.
6. Scholes SFE, Vaillant C, Peacock P, Edwards G, Kelly D. Enteric neuropathy in horses with grass sickness. *Vet Rec*. 1993;132:647-651.
7. Hahn CN, Mayhew IG, de Lahunta A. Central neuropathy of equine grass sickness. *Acta Neuropathol*. 2001;102:153-159.
8. Jago RC, Handel I, Hahn CN, et al. Bodyweight change aids prediction of survival in chronic equine grass sickness. *Equine Vet J*. 2016;48:792-797.
9. Doxey DL, Milne EM, Harter A. Recovery of horses from dysautonomia (grass sickness). *Vet Rec*. 1995;137:585-588.
10. Doxey DL, Johnston P, Hahn C, Reynolds J. Histology in recovered cases of grass sickness. *Vet Rec*. 2000;146:645-646.
11. Pogson DM, Doxey DL, Gilmour JS, Milne EM, Chisholm HK. Autonomic neurone degeneration in equine dysautonomia (grass sickness). *J Comp Pathol*. 1992;107:271-283.
12. Murray A, Pearson GT, Cottrell DF. Light microscopy of the enteric nervous system of horses with or without equine dysautonomia (grass sickness): its correlation with the motor effects of physostigmine. *Vet Res Commun*. 1997;21:507-520.
13. Sanders KM, Kito Y, Hwang SJ, Ward SM. Regulation of gastrointestinal smooth muscle function by interstitial cells. *Phys Ther*. 2016;31:316-326.
14. Hudson N, Mayhew I, Pearson G. A reduction in interstitial cells of Cajal in horses with equine dysautonomia (grass sickness). *Auton Neurosci*. 2001;92:37-44.
15. Hudson N, Mayhew I, Pearson G. Presence of in vitro electrical activity in the ileum of horses with enteric nervous system pathology: equine dysautonomia (grass sickness). *Auton Neurosci*. 2002;99:119-126.
16. Milne EM, Fintl C, Hudson NPH, Pearson GT, Mayhew IG, Hahn CN. Observations on the interstitial cells of Cajal and neurons in a recovered case of equine dysautonomia (grass sickness). *J Comp Pathol*. 2005;133:33-40.
17. Shotton HR, Lincoln J, McGorum BC. Effects of equine grass sickness on sympathetic neurons in prevertebral and paravertebral ganglia. *J Comp Pathol*. 2011;145:35-44.
18. McGorum BC, Pirie RS, Eaton SL, et al. Proteomic profiling of cranial (superior) cervical ganglia reveals beta-amyloid and ubiquitin proteasome

system perturbations in an equine multiple system neuropathy. *Mol Cell Proteomics*. 2015;14(11):3072-3086.
19. Jago RC, Scholes S, Mair TS, et al. Histological assessment of β-amyloid precursor protein immunolabelled rectal biopsies aids diagnosis of equine grass sickness. *Equine Vet J*. 2018;50:22-28.
20. Pavone S, Mandara MT. A morphological and quantitative immunohistochemical study of the interstitial cells of Cajal in the normal equine intestinal tracts. *Equine Vet J*. 2010;42:358-366.
21. Campos CF, Cangussú SD, Duz ALC, et al. Enteric neuronal damage, intramuscular denervation and smooth muscle phenotype changes as mechanisms of Chagasic megacolon; evidence from a long-term murine model of *Tripanosoma cruzi* infection. *PLoS One*. 2016;11:e0153038. https://doi.org/10.1371/journal.pone.0153038.
22. Marrs J, Small J, Milne EM, John HA. Liver and biliary system pathology in equine dysautonomia (grass sickness). *J Vet Med A Physiol Pathol Clin Med*. 2001;48:243-255.
23. Setsuie R, Wada K. The functions of UCH-L1 and its relation to neurodegenerative diseases. *Neurochem Int*. 2007;51:105-111.
24. Song S, Jung YK. Alzheimer's disease meets the ubiquitin proteasome system. *Trends Mol Med*. 2004;10:565-570.
25. Rosen KM, Moussa CE, Lee HK, et al. Parkin reverses intracellular beta-amyloid accumulation and its negative effects on proteasome function. *J Neurosci Res*. 2010;88:167-178.
26. Wishart TM, Mutsaers CA, Riessland M, et al. Dysregulation of ubiquitin homeostasis and beta-catenin signaling promote spinal muscular atrophy. *J Clin Invest*. 2014;124:1821-1834.
27. Keller BA, Volkening K, Droppelmann CA, Ang LC, Rademakers R, Strong MJ. Co-aggregation of RNA binding proteins in ALS spinal motor neurons: evidence of a common pathogenic mechanism. *Acta Neuropathol*. 2012;124:733-747.
28. Salminen A, Kaarniranta K, Kauppinen A, et al. Impaired autophagy and APP processing in Alzheimer's disease: the potential role of Beclin 1 interactome. *Prog Neurobiol*. 2013;106-107:33-54.
29. Blair PJ, Rhee PL, Sanders KM, Ward SM. The significance of interstitial cells in neurogastroenterology. *J Neurogastroenterol Motil*. 2014;20:294-317.
30. Sanders KM. A case for the interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterol*. 1996;111:492-515.
31. Farrugia G. Ionic conductances in gastrointestinal smooth muscles and interstitial cells of Cajal. *Annu Rev Physiol*. 1999;61:45-84.
32. Gfroerer S, Rolle U. Interstitial cells of Cajal in the normal human gut and in Hirschsprung disease. *Pediatr Surg Int*. 2013;29:889-897.
33. Jain D, Moussa K, Tandon M, Culpepper-Morgan J, Proctor DD. Role of interstitial cells of Cajal in motility disorders of the bowel. *Am J Gastroenterol*. 2003;98:618-624.
34. Ördög T, Hayashi Y, Gibbons SJ. Cellular pathogenesis of diabetic gastroenteropathy. *Minerva Gastroenterol Dietol*. 2009;55:315-343.
35. Hudson NPH, Pearson GT, Kitamura N, Mayhew IG. An immunological study of interstitial cells of Cajal (ICC) in the equine gastrointestinal tract. *Res Vet Sci*. 1999;66:265-271.
36. Farrugia G. Interstitial cells of Cajal in health and disease. *Neurogastroenterol Motil*. 2008;20:54-63.
37. Lornicz A, Redelman D, Horvath VJ. Progenitors of interstitial cells of Cajal in the postnatal murine stomach. *Gastroenterol*. 2008;134:1083-1093.
38. Pasternak A, Szura M, Gil K, Matyja A. Interstitial cells of Cajal—a systematic review. *Folia Morphol*. 2016;75:281-286.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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